Mouse Macrophages Are Permissive to Motile Legionella Species That Fail To Trigger Pyroptosis

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Legionella pneumophila, a motile opportunistic pathogen of humans, is restricted from replicating in the lungs of C57BL/6 mice. Resistance of mouse macrophages to L. pneumophila depends on recognition of cytosolic flagellin. Once detected by the NOD-like receptors Naip5 and IpaF (Nlrc4), flagellin triggers pyroptosis, a proinflammatory cell death. In contrast, motile strains of L. parisiensis and L. tucsonensis replicate profusely within C57BL/6 macrophages, similar to flagellin-deficient L. pneumophila. To gain insight into how motile species escape innate defense mechanisms of mice, we compared their impacts on macrophages. L. parisiensis and L. tucsonensis do not induce proinflammatory cell death, as measured by lactate dehydrogenase (LDH) release and interleukin-1β (IL-1β) secretion. However, flagellin isolated from L. parisiensis and L. tucsonensis triggers cell death and IL-1β secretion when transfected into the cytosol of macrophages. Neither strain displays three characteristics of the canonical L. pneumophila Dot/Icm type IV secretion system: sodium sensitivity, LAMP-1 evasion, and pore formation. Therefore, we postulate that when L. parisiensis and L. tucsonensis invade a mouse macrophage, flagellin is confined to the phagosome, protecting the bacteria from recognition by the cytosolic surveillance system and allowing Legionella to replicate. Despite their superior capacity to multiply in mouse macrophages, L. parisiensis and L. tucsonensis have been associated with only two cases of disease, both in renal transplant patients. These results point to the complexity of disease, a product of the pathogenic potential of the microbe, as defined in the laboratory, and the capacity of the host to mount a measured defense.

Legionella pneumophila is a Gram-negative bacterium that opportunistically infects alveolar macrophages of the mammalian lung. Although 50 species of Legionella, comprising more than 70 serogroups, have been identified, only a subset have been associated with disease (39). The most prevalent cause of disease are serogroup 1 strains of Legionella pneumophila, which account for >75% of cases around the world. Non-L. pneumophila species contribute 5 to 10% of the disease burden, primarily in immunocompromised hosts. For example, the non-L. pneumophila species L. parisiensis and L. tucsonensis were each isolated from two renal transplant patients receiving immunosuppressive therapy (38, 69). Nevertheless, L. parisiensis strains can replicate in the human monocytic U937 cell line, in primary macrophages from C57BL/6 mice or guinea pigs, and in Acanthamoeba castellanii (3, 31, 33, 52). Whether these species use similar strategies to those of L. pneumophila to establish a replication niche or to evade the innate immune system is not known, since neither L. parisiensis nor L. tucsonensis has been studied in detail.

Macrophages are key phagocytic defenders of the innate immune system that scout tissues for foreign materials, including pathogens. An important component of their surveillance are pattern recognition receptors that reside on the macrophage surface or within its cytoplasm. The Toll-like receptors (TLRs), present on the surfaces of many cell types, recognize microbe-associated molecular patterns (MAMPs), such as lipopolysaccharide, peptidoglycan, lipoproteins, microbial nucleic acids, and flagellin (2, 36). Much like the TLRs in function, the nucleotide-binding oligomerization domain (NOD-like) receptors (NLRs) monitor the cytoplasm (2, 78). Detection of microbial products by these receptors initiates a signaling cascade that culminates with the secretion of proinflammatory mediators that recruit other lymphocytes to respond to an infection (78).

NLRs participate as components of a protein complex known as the inflammasome. Several different inflammasomes have been characterized based on the MAMPs that initiate their formation and activation. For example, Nalp1, Nalp3, and the more extensively studied NOD proteins each interact with muramyl dipeptides of peptidoglycan (2, 78). NLR family apoptosis inhibitory protein 5 (Naip5; also called Birc1e), which restricts replication of L. pneumophila in mouse macrophages (21, 23, 80), is composed of three domains: (i) an amino-terminal baculoviral inhibitor of apoptosis repeats, (ii) a central NOD domain, and (iii) carboxy-terminal leucine-rich repeats (LRRs) (32). Studies of other NLR proteins indicate that the LRR region is critical for recognition of microbial products, which then triggers oligomerization of the NLR with other inflammasome components through the NOD domain. Subsequently, activation of downstream signaling is carried out by the amino-terminal domains, such as the caspase recruitment domains (32). Unlike the NODs, the Naips and Naip5 control posttranslational processing and secretion of the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 (43). Once formed, the inflammasome recruits and activates caspase-1, which in turn processes pro-IL-1β and pro-IL-18 into their...
mature forms before their release into the extracellular space. Recruitment and activation of caspase-1 are mediated directly or through adapter proteins, such as ASC and Ipaf (1, 85). This proinflammatory reaction is a key element of pyroptosis, the "fiery" cell death seen in response to infection by the pathogens Salmonella enterica, Shigella flexneri, and L. pneumophila (11).

L. pneumophila serogroup 1 has become a model intracellular organism to study both bacterial replication and host detection of pathogens (64). The pathogen requires a type IV secretion system to establish a replication vacuole in human and mouse macrophages, a key determinant of infection (29, 42, 74). However, mice can restrict L. pneumophila infection when the NLR proteins Naip5 and Ipaf detect flagellin (14, 20, 33, 49, 57, 81, 85). While C57BL/6 mice do not support L. pneumophila replication beyond the first 24 h (83), this bacterium replicates profusely in macrophages derived from the A/J mouse strain (82). The A/J Naip5 allele is hypofunctional, based on experiments using transgenic complementation, RNA knockdown, and Naip5−/− mutant mice (21, 37, 80). A/J and Naip5 null mutant mice are defective for inflammasome activation during L. pneumophila infection (37, 49, 57). Likewise, L. pneumophila mutants that lack either flagellin (flaA) or type IV secretion (encoded by multiple dot/icm genes) do not stimulate pyroptosis. Whether components of the inflammasome directly bind flagellin is not yet known, but flaA mutant L. pneumophila subverts Naip5-mediated defenses and replicates in C57BL/6 macrophages (49, 57) and human macrophages (73).

Unlike L. pneumophila serogroup 1, many other species of Legionella do replicate in C57BL/6 macrophages. These include strains of L. micdadei, L. bozemanii, L. dumoffii, L. feeliei, L. longbeachae, L. birminghamensis, L. maceachrini, L. sainthelenii, and L. parisiensis, as well as three non-serogroup 1 strains of L. pneumophila (7, 33, 48). With the exception of L. longbeachae (6, 7), the expression of flagellin and type IV secretion by these strains has not been reported. Therefore, we exploited two non-L. pneumophila strains to test the model that mouse macrophages rely on cytosolic flagellin to restrict growth of intracellular Legionella. We show that two motile strains of L. parisiensis and L. tuscunensis evade replication restriction by C57BL/6 murine macrophages, despite the host cells' ability to detect their divergent bacterial flagellin when it contaminates the cytosol. Mouse macrophage restriction of replication correlates with three of the activities of the canonical L. pneumophila Dot/Icm type IV secretion system, namely, sodium sensitivity, late endosome and lysosome evasion, and phagosome perforation.

MATERIALS AND METHODS

Bacterial strains and culture. L. pneumophila Lp03 (flaA kodR rplD), derived from the Philadelphia 1 strain, the dotA mutant (Lp03), and the flaA mutant have been described previously (10, 50). L. parisiensis and L. tuscunensis were gifts from Cary Engleberg (University of Michigan) that were obtained from the Centers for Disease Control and Prevention (59). Species identity was confirmed by Engleberg (University of Michigan) that were obtained from the Centers for Disease Control and Prevention (59). Species identity was confirmed by sequencing (55), the forward primer Legionp_r [5'-GGG(A/G)(A/T)(T)(T)(T)(A)(G)(A)(T)(C)/AG(T)(T)(G)(C)-3'], the reverse primer Legionp_f [5'TC/AG(T)(T)(C)/AT(G)(C)/CC(AG)/CC(AG)(C)/CC(AG)(C)-3'] (where parentheses indicate a mixed-base site), and the Legionella rnp sequence database (http://www.lrgp.org.uk/web /HP/AskelHPAwebStandard/HPAweb_C/119573805138). Additional species confirmation was obtained by blue-white fluorescence (72), which was observed by L.
and the resulting supernatants were discarded. The pellet was resuspended in 1 ml sterile PBS and analyzed by SDS-PAGE and Coomassie blue staining. Protein concentration was determined by the Bradford assay (Pierce). To disassemble flagella and denature the flagellin protein, the CFPs were incubated at 70°C for 5 min. To degrade the flagellin, CFPs were incubated with 1 µg/ml proteinase K (Sigma) for 1 h at 37°C. Proteinase K was then inactivated by incubation at 75°C for 15 min.

**Immunoblotting.** To evaluate flagellin production, CFPs were boiled for 5 min in Laemmli buffer and resolved by SDS-PAGE, and then the samples were either stained with Coomassie blue or transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in BLOTTO (Tris-buffered saline containing 5% nonfat milk) and incubated at 4°C overnight with monoclonal antibody 2A5, specific to *L. pneumophila* flagellin (50), diluted 1:100 in BLOTTO. Membranes were then washed 5 times in TBST and incubated with secondary goat anti-mouse conjugated to horseradish peroxidase (Pierce), diluted 1:10,000 in BLOTTO, for 1 h at room temperature (RT) with shaking. Membranes were washed as before and preincubated with the West Pico enhanced chemiluminescence (ECL) system (Pierce).

**DNA hybridization.** Genomic DNA from each *Legionella* strain was transferred to a positively charged nylon membrane by use of a Bio-Dot SF (Bio-Rad) apparatus. Flagellin-specific probes were labeled with digoxigenin-dUTP (DIG-dUTP) (Roche) by PCR amplification and detected by nonradioactive CDP*<sub>D</sub>* dilution 3-(4-methoxy-1,2-dioxetane-3,2-yl)phenylphosphate; Roche] chemiluminescence detection. Hybridization was performed under low-stringency conditions at 42°C. Films were analyzed using Image J software (NIH).

**Sodium sensitivity.** Bacterial cultures were grown to the phase indicated and plated in serial dilutions on CYET alone or CYET containing 100 mM NaCl. After incubation at 37°C for 4 days, CFU were counted and sodium sensitivity expressed as follows: [(CFU in CYET + NaCl)/(CFU in CYET)] × 100.

**Immunofluorescence microscopy.** The interaction between *L. pneumophila* and late endosomes and lysosomes was assessed by studying colocalization of the *L. pneumophila* phagosome with LAMP-1 (17). A total of 2.0 × 10<sup>5</sup> macrophages per 12-mm glass coverslip were exposed to PE-phase *Legionella* at an MOI of 0.5 in RPMI-FBS, centrifuged at 200 × g for 5 min at RT, and then incubated at 37°C for 1.5 h in RPMI-FBS containing 100 µg/ml thymidine. After two rinses with 37°C RPMI-FBS, cultures were fixed with 37°C periodate-lysine-parafomaldehyde (44) containing 4.5% sucrose for 30 min at RT. LAMP-1 was stained by rat monoclonal antibody 1D4B at a dilution of 1:500 followed by Oregon Green-conjugated anti-rat serum (Molecular Probes) diluted 1:2,000, bacteria were labeled using an *L. pneumophila*-specific antibody (a kind gift of Ralph Isberg [10]) diluted 1:2,000 followed by Texas Red-conjugated anti-rabbit serum (Molecular Probes) diluted 1:2,000, and DNA was labeled with 0.1 µM 4′,6-diamidino-2-phenylindole (DAPI) in PBS. Samples were analyzed with a Zeiss Plan-Neofluor objective with a numerical aperture of 1.3. At least 100 vacuoles containing *L. pneumophila* were scored in each of three experiments.

**EtBr permeability.** To minimize the potential contribution of caspase-1-dependent pore formation, the coculture period was limited to 1 h and Naip5 mutant macrophages were obtained from A/J mice (22, 49). Bacteria were cultured to the PE phase until they were highly motile. Macrophages plated at 2.5 × 10<sup>5</sup> per glass coverslip were infected at an MOI of 50 for 1 h. Coverslips were inverted onto a 5-µl drop of PBS containing 25 mg/ml ethidium bromide (EtBr) and 5 mg/ml acridine orange placed on the surface of a glass slide. Samples were scored immediately by fluorescence microscopy as described previously (35).

**Statistics.** The significance of quantitative differences was determined using a one-way analysis of variance (ANOVA).

**RESULTS**

**Flagellated strains of *L. parisiensis* and *L. tucsonensis* replicate in C57BL/6 macrophages.** The innate immune system of C57BL/6 mice restricts *L. pneumophila* by detecting cytosolic flagellin (37, 49, 57). Like *L. pneumophila*, when cultured to the stationary phase, *L. parisiensis* and *L. tucsonensis* displayed rapid, directed movement that was apparent by light microscopy. To investigate whether other flagellated species of *Legionella* are restricted by mouse macrophages, we compared the intracellular growth of *L. parisiensis* and *L. tucsonensis* to that of wild-type and flaA mutant *L. pneumophila*. Macrophages were infected with stationary-phase bacteria, and then replication was assessed at 24-h intervals by quantifying CFU. As expected, the number of *L. pneumophila* organisms increased 10-fold over the first 24 h, but subsequently, the infection was suppressed (Fig. 1). In contrast, the yield of *L. parisiensis* increased 100-fold over a 2-day period in primary C57BL/6 macrophages, a pattern similar to that of nonflagellated *L. pneumophila flaA* mutants and consistent with their capacity to replicate in a variety of professional phagocytes (3, 31, 33, 52). Likewise, motile *L. tucsonensis* effectively infected C57BL/6 macrophages, replicating to CFU yields similar to those of *L. parisiensis*. Therefore, evasion of the innate defenses of C57BL/6 macrophages is not unique to nonflagellated bacteria, since two motile strains of non-*L. pneumophila* Legionella species established robust infections.

**L. parisiensis and L. tucsonensis do not trigger pyroptosis.** *L. pneumophila* elicits pyroptosis by a mechanism that requires flagellin, since flaA mutants are not cytotoxic and do not induce IL-1β secretion from macrophages (49, 57). To assess whether flagellated *L. parisiensis* and *L. tucsonensis* fail to trigger an innate immune response, we analyzed macrophage cytotoxicity and secretion of the proinflammatory cytokine IL-1β. When infected for 1 h at a high MOI, PE-phase *L. pneumophila* was cytotoxic; fewer than 35% of macrophages were viable (Fig. 2A). In contrast, nearly 100% of mouse macrophages cultured with *L. parisiensis* and *L. tucsonensis* were viable, a pattern similar to that for macrophages incubated with flagellin-deficient flaA mutants of *L. pneumophila*. Fur-

![FIG. 1. Flagellated strains of *L. parisiensis* and *L. tucsonensis* evade C57BL/6 macrophage restriction of replication. The intracellular growth in C57BL/6 macrophages of *L. pneumophila* (C), flaA mutant *L. pneumophila* (D), *L. parisiensis* (○), and *L. tucsonensis* (△) was determined by quantifying CFU at the times shown and dividing by the CFU associated with cells at 2 h. The values shown are the means ± standard deviations (SD) calculated for triplicate samples in one experiment that is representative of three or more independent trials.](http://iai.asm.org/.../2017 by guest http://iai.asm.org/ Downloaded from)
thermore, after a 2-h incubation, *L. parisiensis* and *L. tucsonensis* triggered secretion of only negligible amounts of IL-1β from macrophages, whereas *L. pneumophila* induced the release of 1,500 pg/ml (Fig. 2B). Increasing the incubation period or the MOI had no effect on the amount of IL-1β secreted (data not shown). Therefore, *L. parisiensis* and *L. tucsonensis* fail to induce pyroptosis in mouse macrophages, despite their motility.

Flagellins from *L. parisiensis* and *L. tucsonensis* are divergent. To verify that *L. parisiensis* and *L. tucsonensis* encode flagellin, we used conventional methods to isolate flagellin from *L. pneumophila* and then analyzed the crude preparations (CFPs) by SDS-PAGE and Western blot analysis. *L. parisiensis* and *L. tucsonensis* produce proteins consistent with the molecular size of *L. pneumophila* flagellin (Fig. 3A). However, CFPs from *L. parisiensis* and *L. tucsonensis* did not react with anti-*L. pneumophila* flagellin antibody (Fig. 3B), indicating divergence at the protein level. A recent DNA array study of several *Legionella* species also reported divergence of flaA in *L. parisiensis* and *L. tucsonensis*, since probes representing the flaA locus of three *L. pneumophila* strains did not hybridize to *L. parisiensis* or *L. tucsonensis* genomic DNA (15). Indeed, several attempts using conventional and degenerate PCR to amplify the complete flagellin gene from these species were unsuccessful. To estimate the extent of this divergence, we generated a labeled probe for each of three regions of *L. pneumophila* flaA and analyzed the probe homology to genomic DNA by dot blot hybridization. *L. parisiensis* DNA hybridized weakly at the N terminus and the core region, whereas *L. tucsonensis* was most divergent in the core region (Fig. 3C). Significant homology was observed for *L. parisiensis* and *L. tucsonensis* in the C-terminal region, as >90% hybridization was evident in the region that also harbors the *L. pneumophila* flagellin carboxy-terminal “death” domain (37). However, a smaller probe designed to encode the final 51 amino acids of *L. pneumophila* flagellin (bp 1275 to 1428) consistently showed

FIG. 2. *L. parisiensis* and *L. tucsonensis* strains do not trigger cell death or IL-1β secretion. (A) C57BL/6 macrophages were incubated for 1 h with twofold dilutions of *L. pneumophila* (●), flaA mutant *L. pneumophila* (▲), *L. parisiensis* (●), and *L. tucsonensis* (●), and viability was quantified by Alamar Blue reduction. Results from one experiment, representative of three, are shown. (B) Macrophages pretreated with LPS were infected with the strain indicated for 2 h, and secreted IL-1β was quantified. Results shown are means ± standard errors (SE) for duplicate wells in three experiments.

FIG. 3. Characterization of crude flagellin preparations. Flagellin samples prepared in parallel from broth cultures were separated by SDS-PAGE and analyzed by Coomassie blue staining (A) of *L. pneumophila* (lane 2), flaA mutant *L. pneumophila* (lane 3), *L. parisiensis* (lane 4), and *L. tucsonensis* (lane 5) or Western analysis, using anti-*L. pneumophila* flagellin antibody (B), of *L. pneumophila* (lane 1), flaA mutant *L. pneumophila* (lane 2), *L. parisiensis* (lane 3), and *L. tucsonensis* (lane 4). Positions of molecular mass standards are shown (lane 1 in panel A). (C) Relative genomic hybridization under low-stringency conditions with DNA probes for the indicated regions of *L. pneumophila* flaA.
less hybridization for *L. parisiensis* and *L. tucsonensis* genomic DNA than for *L. pneumophila* DNA (data not shown). Therefore, by analogy to escape from TLR5 recognition by several species of motile bacteria (4), it remained formally possible that the divergence observed for the flagellin genes and proteins of *L. parisiensis* and *L. tucsonensis* was sufficient to circumvent detection by the cytosolic surveillance machinery.

**Cytosolic *L. parisiensis* or *L. tucsonensis* flagellin induces proinflammatory cell death.** Pyroptosis in murine bone marrow-derived macrophages is induced when flagellin that presumably has leaked to the cytosol is detected by Naip5 and Ipaf (37, 49, 57). To determine if the divergent *L. parisiensis* and *L. tucsonensis* flagellins elude detection, we introduced crude flagellins from broth-grown cultures into the cytosol and then tested if either could trigger pyroptosis. C57BL/6 mouse macrophages were incubated for 2 h with the protein transfection reagent Profect P1 complexed with CFP obtained from *L. pneumophila*, *L. parisiensis*, or *L. tucsonensis*, and then the LDH released by intoxicated cells was quantified. Mouse macrophages whose cytosol was transfected with CFPs from *L. pneumophila*, *L. parisiensis*, and *L. tucsonensis* each released >80% of their LDH, significantly more than the quantities measured for transfection reagent alone and the flaA mock prep (*P* < 0.0001) (Fig. 4A). The toxicity was due to protein and not some other bacterial product in the CFP, since treatment of the CFP with proteinase K prior to transfection reduced the toxicity (Fig. 4A, gray bars).

A hallmark of pyroptosis is secretion of IL-1β, a result of caspase-1 activation. Not only did the cytosolic CFPs trigger cell death, but each also induced secretion of IL-1β from macrophages. When macrophages were transfected with CFP, ~70% more IL-1β was secreted from those macrophages than from those transfected with CFP pretreated with proteinase K (Fig. 4B, gray bars), and >95% more was secreted than that from macrophages that were treated with *L. pneumophila* flaA mock CFP or that from the negative control samples (Fig. 4B).

As another approach to ascertain whether pyroptosis was primarily a response to the flagellin protein in the CFP or instead a response to some other microbial contaminant, we analyzed macrophages that lacked both Naip5 and Ipaf, the NLRs that collaborate to detect cytosolic flagellin (37, 49, 57). As observed previously for *L. pneumophila* (49), in response to cytosolic CFPs prepared from *L. parisiensis* or *L. tucsonensis*, C57BL/6 macrophages secreted >60% of their LDH (Fig. 4C, black bars), whereas Naip5- and Ipaf-deficient macrophages released significantly less (*P* < 0.01) (Fig. 4C, gray bars). The *L. tucsonensis* CFP elicited more LDH release from the Naip5- and Ipaf-deficient macrophages than did either of the negative

![FIG. 4. Flagellin preparations from *L. parisiensis* and *L. tucsonensis* trigger macrophage cell death. To determine whether flagellins from *L. parisiensis* and *L. tucsonensis* trigger pyroptosis, the amounts of LDH (A) and IL-1β (B) released from C57BL/6 macrophages transfected with heat-treated crude flagellin (1.25 μg; black bars) or treated with proteinase K (gray bars) and incubated for 2 h were quantified. The data shown are means ± SE for three experiments, where asterisks indicate statistical differences from flaA mutant *L. pneumophila* (*P* < 0.05 using one-way ANOVA). (C) To determine whether the pyroptosis response to cytosolic flagellin required the NLR proteins Naip5 and Ipaf, LDH released from macrophages derived from C57BL/6 or Naip5−/− Ipaf−/− double mutant mice was quantified as in panel A. Asterisks indicate statistical differences (*P* < 0.01) compared to C57BL/6 macrophages exposed to WT *L. pneumophila*, calculated using one-way ANOVA; the caret indicates that *P* = 0.016.](http://iai.asm.org/article/40287/4)
controls (Profect P1 and L. pneumophila flaA mock CFP), suggesting that in addition to flagellin, other microbial products contributed to the pyroptosis response. Thus, the capacity of L. parisiensis and L. tucsonensis to replicate in C57BL/6 macrophages (Fig. 1) could not be accounted for fully by postulating that the cytosolic surveillance pathway is blind to their flagellins.

**L. parisiensis and L. tucsonensis do not exhibit type IV secretion-dependent traits.** To activate the inflammasome complex and subsequently induce secretion of IL-1β, L. pneumophila requires not only flagellin but also type IV secretion, which is thought to provide a conduit to the cytoplasm (45, 49, 57). Therefore, we next tested whether the cytotoxicity defect of L. parisiensis and L. tucsonensis is attributed to their type IV secretion systems.

Although a mechanism has not been established, L. pneumophila strains that are defective for type IV secretion are resistant to sodium; indeed, several of the dot/icm type IV secretion mutants were originally isolated based on their ability to grow on high concentrations of NaCl (75). Salt sensitivity of an L. parisiensis strain has also been reported (52). Accordingly, as one probe of their type IV secretion systems, we tested the ability of the L. parisiensis and L. tucsonensis strains to form colonies on medium containing 100 mM NaCl. L. pneumophila is sensitive to NaCl in the PE phase of growth, yet exponential-phase bacteria and PE dotA mutants are NaCl resistant (13) (Fig. 5A). In contrast to L. pneumophila, PE-phase cultures of the strains of L. parisiensis and L. tucsonensis were resistant to salt. Compared to the dotA mutant salt-resistant control, the non-L. pneumophila cultures were 10-fold more tolerant to NaCl (Fig. 5A).

A second hallmark of type IV secretion-competent L. pneumophila is the ability to evade the late endosomal compartment, which is rich in LAMP-1 (74) (Fig. 5B). However, like L. pneumophila dotA mutants, >60% of PE-phase L. parisiensis and L. tucsonensis cells colocalized with LAMP-1 within 2 h of internalization by C57BL/6 macrophages (Fig. 5B).

A third characteristic of type IV secretion is the ability to perforate macrophage membranes, an activity evident by staining cells, after a 1-h infection, with the fluorescent dyes EtdBr and acridine orange (35). Cells with intact membranes exclude EtdBr; as such, cells with red nuclei are scored as EtdBr permeable and those with green nuclei are scored as alive or EtdBr impermeable. As shown previously, WT L. pneumophila permeabilized 70% of macrophages, whereas L. pneumophila dotA mutants did not (<10% of macrophages had red nuclei)

**FIG. 5.** L. parisiensis and L. tucsonensis do not display features of the canonical Dot/Icm type IV secretion system. (A) Sodium sensitivity was quantified by plating E- or PE-phase broth cultures on medium with or without 100 mM NaCl and using the formula \((\frac{\text{CFU on CYET+Nal}}{\text{CFU on CYET}}) \times 100\). (B) Colocalization with the late endosomal and lysosomal protein LAMP-1 was quantified by immunofluorescence microscopy after incubating C57BL/6 macrophages for 1.5 h with PE-phase WT L. pneumophila, dotA mutant L. pneumophila, L. parisiensis, or L. tucsonensis. Results shown are means ± SE for three experiments. (C) Permeability of A/J macrophages after incubation for 1 h with PE-phase Legionella was determined by uptake of ethidium bromide. The data shown are means ± SE calculated from three experiments.
Taken together, their phenotypes indicate that *L. parisiensis* strain remained green (Fig. 5C), consistent with the inability of *L. parisiensis* to lyse sheep red blood cells (3). Likewise, the *L. tucsonensis* strain failed to induce macrophage permeability. Taken together, their phenotypes indicate that *L. parisiensis* and *L. tucsonensis* do not express the canonical Dot/Icm type IV secretion system of *L. pneumophila*, which forms pores in macrophage membranes that are thought to provide a conduit for flagellin to the macrophage cytoplasm.

**DISCUSSION**

The response to cytosolic flagellin by the innate immune machinery of C57BL/6 macrophages results in resistance to *L. pneumophila* infection. Yet other species of *Legionella* establish a productive replication niche in these macrophages (3, 7, 33, 48). We show here that the flagellated species *L. parisiensis* and *L. tucsonensis* replicate in C57BL/6 macrophages and that they both fail to stimulate proinflammatory cell death. Pyroptosis is likely one rapid response by C57BL/6 macrophages but also for other components of the immune system, most notably neutrophils, cytokines, and chemokines (24, 25, 37, 45, 47, 49, 54, 76). By genomic hybridization, the macrophage cytosol (45, 49, 57). Likewise, motile *L. pneumophila* organisms replicate in C57BL/6 macrophages but lack pore-forming activity, despite carrying at least some of the dot/icm loci (7). We postulate that without release of flagellin into the cytoplasm, macrophages remain blind to these intracellular *Legionella* organisms and fail to trigger pyroptosis to combat the infection.

Although we have focused here on the early response of macrophages to *L. pneumophila*, host defense is a culmination of many factors. Indeed, wild-type and flaA mutant *L. pneumophila* cells are eventually cleared from the lungs of A/J Naip5 mutant and C57BL/6 mice, respectively, indicating that other immune response pathways contribute to restricting bacterial replication (49). Many studies of infections in animal and human models have shown an important role not only for macrophages but also for other components of the immune system, most notably neutrophils, cytokines, and chemokines (70). For example, gamma interferon (IFN-γ), IFN-ω/β, and tumor necrosis factor alpha (TNF-α) each induce macrophage resistance to *L. pneumophila* (12, 18, 51, 53).

Flagellin is a potent stimulator of innate immune signaling pathways. By analogy to the TLR5 epitope of flagellin, which is conserved across bacterial species, it is likely that the epitope recognized by the NLR cytoplasmic receptors is highly conserved (4, 45, 46, 49, 57, 68, 85). For example, mouse macrophages detect cytosolic flagellins from *Legionella, Salmonella, Bacillus,* and *Pseudomonas* through the NLRs Ipaf and Naip5 (24, 25, 37, 45, 47, 49, 54, 76). By genomic hybridization, the flaA sequences from *L. parisiensis* and *L. tucsonensis* appear to be divergent from the *L. pneumophila* flaA sequence, including in the C-terminal region, which harbors one toxic epitope recognized by Naip5 and required for activation of the inflammasome (37). Nevertheless, *L. parisiensis* and *L. tucsonensis* flagellins can trigger pyroptosis when delivered directly to the cytosol (Fig. 4). Therefore, the divergence of the *L. parisiensis* and *L. tucsonensis* flagellin species that is evident at the DNA and protein levels is not sufficient to account for the lack of cytotoxicity of these bacteria (Fig. 2). Instead, we favor the model that during *L. parisiensis* or *L. tucsonensis* infection, flagellin does not escape from the vacuole to contaminate the cytoplasm.

Bacterial secretion systems are one route to the cytosol for bacterial products (5, 30, 71). For example, *Salmonella* activates the innate immune system not only through TLRs but also through NLRs, since mutants that lack a type III secretion system do not activate NLR signaling (40, 41). Therefore, the cytosolic surveillance system provides macrophages a mechanism to respond specifically to invading pathogens that express virulence factors that breach the phagosome, namely, toxins and specialized secretion systems (11, 19). The cytosolic surveillance system detects *L. pneumophila* bacteria that have a functioning type IV secretion apparatus (49, 57), which the bacteria require to establish replication vacuoles (74). Although translocation of flagellin by *L. pneumophila* through its type IV secretion system has not been demonstrated unequivocally, it is hypothesized that as for *Salmonella*, when virulence effectors are secreted, minute amounts of flagellin are also translocated from the bacterium to the macrophage cytosol (37, 46, 66). Compared to *L. pneumophila*, *L. parisiensis* and *L. tucsonensis* lack phenotypes characteristic of the canonical type IV secretion system.

It remains to be determined whether *L. parisiensis* and *L. tucsonensis* evade detection by the inflammasome due to either incompatibility of their divergent flagellin species with the secretion system, a pore size that is not permissive for flagellin to escape, or a defective secretion system. Genomic DNA hybridization studies of different *Legionella* strains indicate that some, but not all, of the many dot/icm structural and secreted effector genes are conserved (3, 15; C. Buchrieser, personal communication). In particular, *L. parisiensis* genomic DNA hybridizes to icmD, icmF, dotF, dotE, and lidA, and *L. tucsonensis* contains sequences similar to icmD, dotL, dotN, dotD, dotC, and dotB (Buchrieser, personal communication). Accordingly, some *L. pneumophila* Dot/Icm components are either highly divergent or absent in *L. parisiensis* and *L. tucsonensis*. In some non-*L. pneumophila Legionella* species, the capacity to replicate intracellularly or to transfer plasmids by conjugation does not correlate with sodium sensitivity (34, 52), a trait conferred by the Dot/Icm type IV secretion system (75). Furthermore, under certain conditions, the canonical Dot/Icm type IV secretion system is dispensable. When amoebae are infected with water- or Ers-treated dot/icm mutants, the bacteria replicate intracellularly and avoid phagosomal acidification as efficiently as the wild-type parental strain, provided that they encode the homologous Lyh type IV secretion system (8, 9). *L. pneumophila* also utilizes a type II secretion system to replicate proficiently in host cells (60). Thus, legionellae utilize a variety of specialized secretion systems to parasitize macrophages. Some, but not all, of these machineries may inadvertently translocate flagellin to the cytoplasm of host cells.

In humans, *L. pneumophila* infection causes an acute pneumonia and severe inflammation, where lung exudates contain macrophages, polymorphonuclear cells, fibrin, red blood cells,
proteinaceous material, and cellular debris indicative of cell death and cell lysis (28, 79). In mouse macrophages, both flagellin and type IV secretion are key inducers of the inflammatory response to \textit{L. pneumophila}, since the Dot/Icm type IV secretion system can lyse white blood cells, flagellin is proinflammatory, and both factors are required for activation of the inflammasome (35, 49, 50, 58, 61–63, 65, 73). Likewise, studies of both human peripheral blood-derived monocytes and an alveolar epithelial cell line indicate that a Naip- and Ipaf-mediated response to flagellin reduces the yield of intracellular \textit{L. pneumophila} (73). Accordingly, it is plausible that despite their capacity to replicate in a variety of host cells (3, 31, 52), \textit{L. parisiensis} and \textit{L. tucsonensis} are less frequent causes of Legionnaires’ disease because their flagellin is not translocated to the macrophage cytoplasm. By this model, the inflammation and cellular damage characteristic of Legionnaires’ disease are manifestations of not only bacterial replication but also robust activation of the inflammasome and extensive pyroptosis triggered by flagellin that contaminates the cytosol during type IV secretion. However, it cannot be assumed that mouse and human macrophages utilize similar mechanisms to restrict infection. For example, the human Naip protein has been shown to interact with caspases 3, 7, and 8, whereas mouse Naip5 is predicted to regulate caspase-1 activity (reviewed in reference 70). As another test of this model, it would be important to analyze multiple isolates of each non-L. pneumophila species, due to well-documented heterogeneity within species and the genus (3, 6, 7, 15, 16, 26, 27, 33, 34, 48, 56, 77).

Our analysis of non-L. pneumophila Legionella species has extended the evidence that pore formation by canonical type IV secretion makes \textit{L. pneumophila} vulnerable to early detection of flagellin by the innate immune system. It is also notable that the potential of legionellae to cause human disease appears to be inversely correlated with the capacity to replicate in macrophages of C57BL/6 mice (3, 7, 33, 39, 48). Accordingly, this study points to the complexity of human disease, a product of both microbial virulence traits, as defined by laboratory assays, and the capacity of the host to mount a measured immune response.

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